

# Physiology of Bone: Mineral Compartment Proteins As Candidates for Environmental Perturbation by Lead

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Termine et al. first demonstrated that sequential dissociative extraction and fractionation procedures with protease inhibitors could provide a convenient approach for the study of mineral compartment constituents. The primary extraction regimen used 4 M guanidine HCl to remove most of the protein from the nonmineralized phase of bone. Subsequently, EDTA-guanidine was used to remove the mineral-phase components. These methods discriminate on the basis of physical-chemical association with a mineral phase rather than on the specific gene products of a particular cell. In the present discussion emphasis is directed at a group of divalent cation binding proteins isolated from the mineral compartment of bone. The localization, synthesis, and chemical characteristics of osteonectin, bone sialoproteins I and II, and bone acidic glycoprotein-75 are discussed and offered as possible sites for perturbation by the environment with lead exposure.

## Introduction

The cumulative effects of exposure to an environment can be expressed as alterations in cellular products and can be preserved in the mineralized tissues of an organism. To this end, archeological specimens and skeletal rudiments have been shown to harbor elements common to an organism's exposure. Included among these elements is the inorganic element lead. The reliability of the record maintained within these tissues depends in great part upon the degree of remodeling to which the matrices are subjected and the site from which the samples are obtained (1). Bone is a complex tissue consisting of mineral, cells, and an extracellular matrix composed of collagenous and non-collagenous proteins, all of which are subject to processing as a function of development and growth.

There is considerable evidence that noncollagenous matrix proteins associated with bone are important in the regulation of bone homeostasis, although the precise role of specific proteins has not yet been elucidated (1-4). Our understanding of the matrix components of mineralized tissue have benefitted from advances in

extraction and fractionation procedures performed in the presence of protease inhibitors (5). In essence, primary extraction of mineralized matrices with dissociative agents such as 4 M guanidine HCl removes many of the proteins from the mineral phase of these matrices. Subsequent extraction with guanidine HCl/EDTA dissolves the mineral phase and extracts the strongly mineral binding noncollagenous proteins considered to have important functions in maintaining bone homeostasis. Thus, many studies have been directed at understanding the properties of these proteins.

For example, in attempts to understand the effects of these extracellular matrix proteins on the osteogenic process, a demineralized matrix, containing all the factors necessary for mineralization, has been used (6-8). In this system, demineralized bone matrix power implanted subcutaneously in rats induces new bone formation in a sequential manner analogous to fracture healing (9). After the initial influx of polymorphonuclear leukocytes, mesenchymal cells are attracted to the site, where they subsequently attach, spread, proliferate, and synthesize the appropriate matrix required for the formation of cartilage and bone. The fact that an initial event in this process is the migration, followed by attachment of cells to the site, strongly supports the concept that migration and attachment are directed via specific signals. In support of this, several chemotactic factors (10-13), as well as attachment proteins, have been identified in guanidine/EDTA extracts of bone (14-18).

In addition, several factors and proteins extracted from mineralized tissues, e.g., bone morphogenic pro-

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tein, growth factors, and osteonectin, have been implicated as having a role in initiation of mineralization (1-5). In this article the properties of bone-associated attachment proteins are discussed. Also, osteonectin/SPARC/BM-40, a protein first identified in bone (5,19), is reviewed. These proteins were selected not only because of their potential importance to maintaining mineral homeostasis, but also because of their potential to be modulated by cationic metals, such as lead. All the proteins discussed below are cation-binding proteins requiring solvents containing EDTA for extraction.

## Osteonectin/SPARC/BM-40

Osteonectin is a 32-kilodalton (kDa) glycoprotein composed of a single polypeptide chain with several intramolecular disulfide bonds (19-21). This protein is one of the principal noncollagenous proteins of bone (19-22). Osteonectin is synthesized by osteoblastlike cells in culture (23,24) and is secreted as an extracellular matrix protein in the cell layer (26,27). *In vitro* binding studies have revealed that osteonectin has a high affinity for calcium ( $K_d = 3 \times 10^{-7}$  M), hydroxyapatite ( $K_d = 8 \times 10^{-8}$  M), and collagen (20,21,28). The affinity of osteonectin for hydroxyapatite is six times stronger than that of bone Gla protein (BGP, also called osteocalcin) (25). These data have been interpreted to indicate that osteonectin may have an important function in bone (1). Similar, if not identical, proteins have been derived from nonmineralized tissues and by sequence analysis shown to have high homology with the protein derived from bone (26). Stenner et al. (27) have described an analogous protein in platelets, and Dzidek et al. (28) identified a basement membrane protein (BM40) from mouse EHS tumors that appears identical to osteonectin. The protein termed SPARC (secreted protein, acidic rich in cysteine) derived from mouse parietal endoderm (29,30) manifests significant homology with the bone-derived protein as does a 43 kDa protein secreted by bovine endothelial cells in culture (31). The presence of these proteins in nonmineralizing tissues queries the possible physiological role that these molecules play in nonosseous matrices. The mineral binding properties of osteonectin make it an important candidate for environmental perturbations with other cations.

Bolander et al. (26) have recently sequenced the bovine osteonectin molecule. The nucleotide sequence obtained from these studies revealed that osteonectin contains 304 amino acids, with a 17-residue signal peptide. Structural analysis deduced from this sequence indicated that osteonectin contains at least four domains. The amino terminus of the protein appears to harbor a potential hydroxyapatite binding site. The next domain, a cysteine-rich area, has a sequence homology with the cysteine-rich domain in turkey ovomucoid, bovine acrosyn inhibitor, and bovine pancreatic trypsin inhibitor. The carboxyl half of the protein

contains two sequence homologies with central calcium-binding loops, "EF hands," with the potential of being high-affinity calcium binding sites (26).

Recently, McVey et al. (32) have shown that the SPARC/osteonectin gene contains 10 exons and covers 26.5 kilobase pairs of DNA. Findlay et al. (33) extended these studies and demonstrated that interspecies differences probably reside in a exon at the N-terminus. Analyses of the remaining exons revealed that two N-terminal glutamic acid-rich sequences predicted to undergo conformational change upon binding of calcium and the EF-hand  $\text{Ca}^{2+}$  binding domain are each encoded by a single exon. It was concluded that the SPARC gene probably has not evolved by shuffling of exons from other  $\text{Ca}^{2+}$  binding proteins (33). It is noteworthy that when the 5' flanking region of the SPARC/osteonectin gene was placed in front of the bacterial chloramphenicol acetyltransferase gene, neither TATA nor CCAAT box sequences were detected. However, unlike most other genes lacking these motifs, mapping of the 5' end of the SPARC/osteonectin gene by RNase protection and primer extension analysis revealed only a single major and one minor transcription start site (32). Young et al. (34) noted that heterogeneity observed at the 5' end of the gene may be due to a lack of a CCAAT or TATA box believed to be involved in positioning of RNA polymerase (35). In addition, nine sequence motifs have been identified in the osteonectin gene (34). These motifs include a) TAAT associated with growth hormone responsiveness (36); b) GAANNNTTC, a heat shock element (37); c) TCAGAG, responsible for cAMP action (39); d) TGCCTC, regulating metal responsiveness (39); e) CCAAT, associated with CCAAT protein binding (40); f) TCGNNNGCCAA, NFI bind-

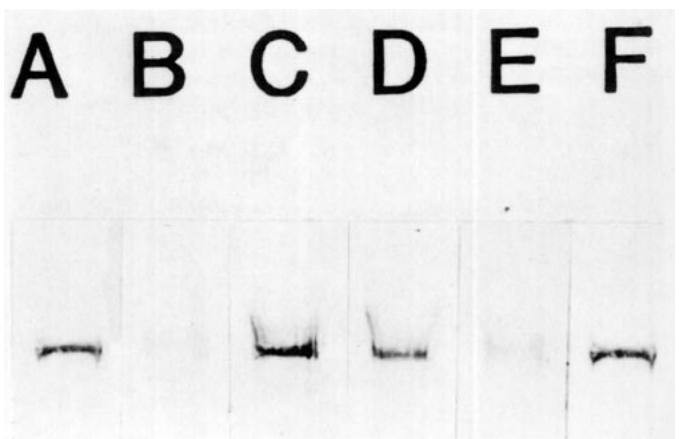


FIGURE 1. Western blot using rabbit-anti-bovine osteonectin antibodies (courtesy of L. W. Fisher, NIH/NIDR). (A) Control cells grown on tissue culture plates. (B) Osteoligament cells treated with 5 mM L-azetidine-2-carboxylic acid. (C) Osteoligament cells treated with 43°C heat for 90 min. (D) Canine ligament cells exposed to 100 mM sodium arsenite for 1.5 hr. (E) Osteoligament cells challenged with 45°C heat for 30 min. (F) Canine ligament cells grown in the presence of ionophore A23187 for 8 hr.

ing (41); g) GC box, SP1 binding (42); h) an imperfect palindrome as a possible stem loop structure; and i) GAGA box, a S1-one endonuclease recognition site (34).

We have demonstrated that although the osteonectin gene contains a heat shock element, osteoligament cells challenged with 45°C for 30 min do not respond with enhanced mRNA production. Interestingly, the levels of mRNA remain stable and the cellular levels of protein synthesis increase 2-fold. In contrast, L-azetidine-2-carboxylic acid, an amino acid analog, known to induce a variety of stress and heat shock proteins, lowers both cellular cells of osteonectin (Fig. 1) and its mRNA. Sodium arsenite significantly enhances osteonectin mRNA levels but depresses cellular levels of the protein, suggesting that stability of the message may be altered with exposure to these agents.

In addition to these agents, a number of environmental and pharmacological agents have been shown to affect the expression of osteonectin/SPARC. Retinoic acid and dibutyryl-cAMP have been shown to enhance the production osteonectin and mRNA 40-fold (29). Low doses of tumor-derived growth factor has been shown to increase osteonectin mRNA in ROS 17/2 cells 2- to 3-fold (36). Conversely, parathyroid hormone decreased osteonectin mRNA levels in UMR-106-01 osteosarcoma cells (34), while 1,25-dihydroxyvitamin D<sub>3</sub> appears to down regulate its expression in cultured human bone cells (43). Finally, viral transformation has been shown to drastically reduce osteonectin mRNA levels in chick (44), mouse, and a number of tumors (30).

Thus, all evidence to date indicates that osteonectin is under tight regulation and is associated with areas of rapid tissue remodeling (45,46). As such, the protein or its mRNA have been localized in the stroma of breast and gastric carcinomas, the basement membranes about tumor islands, and in expanding human decidua (47).

## Attachment Proteins

Attachment proteins, by modulating cell-cell or cell-substratum interactions, alter diverse biological processes including embryonic development, wound healing, tumor cell metastasis, and tissue regulation. The potential significance of such proteins in the regeneration of cell behavior has resulted in increasing efforts to identify and understand the properties of attachment proteins (48,49). A common RGD (arg-gly-asn)-peptide sequence has been identified in the region associated with cell attachment in several of these proteins, e.g., fibronectin, vitronectin, bone sialoprotein I, and bone sialoprotein II (49). In addition, the ability of these same proteins to promote cell migration may be regulated through the domain containing the RGD-peptide sequence (49,50). Attachment of cells to such proteins involves interactions with specific cell surface glycoprotein receptors. While these receptors are dis-

tinct, they have been shown to be structurally and functionally related, belonging to a superfamily of receptors called integrins. Structurally these receptors appear similar, containing heteromeric complexes of noncovalently associated alpha and beta subunits (48,49). The integrin receptors require divalent cations to function; that is in the absence of divalent cations, the receptor cannot bind to its ligand (51). Thus, any metal, e.g., lead, that has the potential to alter receptor-ligand interactions may prevent cell attachment and spreading.

While proteins that alter the attachment properties of cells have been identified in tissues other than bone, it is not until recently that such proteins have been identified in bone. Initial studies in our laboratory showed that bone cells, unlike Chinese hamster ovary (CHO) cells, become attached to plates preincubated with fibronectin-free serum, leading us to speculate that bone cells synthesize a protein that modulates cell attachment (52). We also demonstrated that guanidine/EDTA extracts of demineralized tissues promote attachment of gingival fibroblasts *in vitro* (53). More recently, in collaboration with W. Butler and C. Prince, we identified two fractions, after DEAE-Sephacel chromatography of rat bone EDTA extract, that markedly promoted attachment and spreading of gingival fibroblasts (16). After further purification from rat bone (54) and human developing bone (20), these proteins, identified as bone sialoprotein I (BSP I) and as bone sialoprotein II (BSP II)\* were shown to have attachment activity (16-18,55,56). Discussed below are some characteristics and properties of BSP I and BSP II. Also discussed is bone acidic glycoprotein-75 (BAG-75), a protein isolated from rat bone, reported to have attachment activity (14). All three attachment proteins are sialic acid-rich phosphorylated glycoproteins.

Sialic acid-rich noncollagenous proteins of bone were first identified in the 1950s as a direct result of attempts to detect molecules in bone that bind radioactive elements from the atomic bomb test fallout. Vaughn (57) showed that plutonium was located in areas of bone staining with periodic acid-Schiff (PAS), suggesting binding to noncollagenous molecules. Subsequently, Herring and Kent (58) isolated and characterized a cation-binding glycoprotein from adult bovine bone. This protein, which they called bone sialoprotein, was 20% by weight sialic acid and had a molecular weight of approximately 25,000 Da. Two decades later, using protease inhibitors and denaturing solvents, Fisher et al. (20,59) showed that the 25 kDa protein was a degradation product of a larger protein, BSP II.

BSP II has been purified and partially characterized from developing calf bone (59), bovine bone (60), rat bone (15), and developing human bone (20). The intact

\*Throughout this article BSP I is used to designate bone sialoprotein I, also called osteopontin, 44 kDa bone phosphoprotein, 2ar, Sppl and tumor-secreted phosphoprotein. BSP II is used to designate bone sialoprotein II, also called BSP (bone sialoprotein). BAG-75 is used to designate bone acidic glycoprotein-75.

protein, purified from bovine and human tissues, is 70 to 80 kDa on SDS gels stained with Alcian blue. BSP II does not stain with Coomassie blue unless the sialic acid residues are removed with neuraminidase (20). Human BSP II contains approximately 50% protein, 12% sialic acid, 7% glucosamine, and 6% galactosamine. Rat BSP II appears to have a lower molecular weight, 57 kDa, and contains approximately 5% sialic acid (60). DNA sequence analysis indicated that BSP II contains an RGD-peptide sequence (15), which presumably is responsible for its ability to promote cell attachment (15,18). Except for this conserved RGD-peptide sequence, BSP II shows no structural similarity to BSP I. The region surrounding the RGD-peptide sequence was shown to be similar to the RGD region in vitronectin (15). This includes 9 identical residues and 3 conserved substitutions in a region of 24 residues. No homologies in this region to other attachment proteins were reported. Preliminary studies on the localization of BSP II in tissues by *in situ* hybridization studies indicate that BSP II is more selective for bone tissues, when compared with BSP I (M. Young, personal communication).

In our laboratories we have been evaluating BSP II for its attachment activity for cells of periodontal origin. Cells of the periodontium are considered to have the capacity to stimulate regeneration of the periodontium, e.g., bone, cementum, and dentin (61). BSP II promotes gingival fibroblast attachment and RGD-peptides block this activity (18). Also, a keratin sulfate proteoglycan isolated from rabbit bone, where the core protein is known to be BSP II (62), enhanced fibroblast cell attachment. Pretreatment of this proteoglycan with keratinase enhanced this activity further (unpublished data). BSP II does not promote attachment of A431 epithelial cells (18). Oldberg et al. (63) isolated the cell receptor for BSP II using osteosarcoma cells, ROS 17/2.8, cells known to exhibit enhanced attachment in the presence of BSP II (15). This protein receptor is remarkably similar if not identical to a previously characterized vitronectin receptor (64) and binds both vitronectin and BSP II. Further studies directed at determining if other bone cells, e.g., osteoclasts, nontumorigenic osteoblasts, have specific receptors for BSP II and will provide information of the specificity of BSP II receptor interactions.

Bone sialoprotein I was the first bone protein to be recognized as having attachment activity and has been given several different names, which include *a*) 44 kDa bone phosphoprotein (44 kDa BPP), based on molecular weight sedimentation equilibrium (54), *b*) osteopontin, suggesting a bridging potential between cells and mineral in bone (15); *c*) 2ar, a tumor promoter-inducible protein secreted by mouse JB6 epidermal cells (65), now recognized as the murine homologue of rat BSP I (66); *d*) mouse secreted phosphoprotein I (Spp I) (67); and *e*) tumor-secreted phosphoprotein. Most recent evidence suggests that a phosphoprotein,  $\approx$  62 K (varying depending on species), secreted by spontaneously transformed cells (68,69) is related if not identi-

cal to BSP I. Elevated secretion of this protein was reported for tumor cells (68,69) as well as plasma and sera from patients with disseminated carcinomas and with gram negative sepsis (68).

BSP I isolated from a variety of mineralized tissues, e.g., rat bone (15), developing calf bones (59), and developing human bone (20), appear to be very similar to each other. BSP I ( $\approx$  68,000 molecular weight depending on species) is a glycoprotein, rich in aspartic acid and glutamic acid, containing 12 residues of phosphoserine and one of phosphothreonine (54). cDNA sequencing studies indicate that this protein contains an RGD cell attachment sequence (15). Carbohydrate, approximately 17% of the mass of the molecule, including about 10 residues of sialic acid, is present as 1 *N*-glycoside and 5 to 6 *O*-glycosides (54). Secondary structure analysis by Prince et al. (70) indicate BSP I, like osteonectin, contains an EF hand, representing a calcium binding site. As predicted for fibronectin, the cell attachment site in BSP I is located in a beta turn. BSP I also has a run of 9 aspartic acid residues (70) suggested to contribute to its tight binding to hydroxyapatite (15). Young et al. (71) have shown that the genes for both human BSP I and human BSP II are located on chromosome 4, while Fet et al. (67) localized the mouse gene for BSP I to chromosome 5.

Prince et al. (72) established that osteosarcoma cells, ROS 17/2.8 cells, synthesize and secrete BSP I. Subsequent studies showed that BSP I production by bone cells *in vitro* can be modulated by several factors. The production of BSP I and BSP I mRNA are enhanced in ROS 17/2.8 cells exposed to 1,25-dihydroxyvitamin D<sub>3</sub> (72,73). In addition, transforming growth factors beta (74), glucocorticoids (73), and parathyroid hormone regulate expression of BSP I by bone cells (74). Studies involved with evaluating the expression of BSP I by other cells indicated that in epidermal cells 12-*O*-tetradecanoylphorbol-13-acetate (TPA) increases the expression of this protein. *In vitro* tumorigenic cells secrete 10-fold or more BSP I when compared with similar nontumorigenic cells (66,68,69). These studies implicate this protein as having a role in regulating tumor growth *in vivo*.

Immunohistochemical analysis (75-77) and *in situ* hybridization studies of BSP I or its mRNA indicate that this protein is present in bone, osteoid, and osteoblasts, as well as cells considered to be precursors to osteoblasts or cells of hemopoietic origin. High levels of BSP I or its mRNA are also observed in kidney, secretory epithelium, and ganglia of the developing ear, in brain, and in bone marrow-derived granulated material gland cells in the uterus and deciduum (46,78). Thus, it is evident that the biochemical functions of BSP I are not limited to mineralized tissues.

Independent studies by Somerman et al. (16,79,80) and Oldberg et al. (15) indicated that BSP I promotes the attachment and spreading of osteoblastlike cells, as well as a variety of other cell types *in vitro*. Detailed studies on the attachment properties of BSP I suggest properties particular to this protein when compared

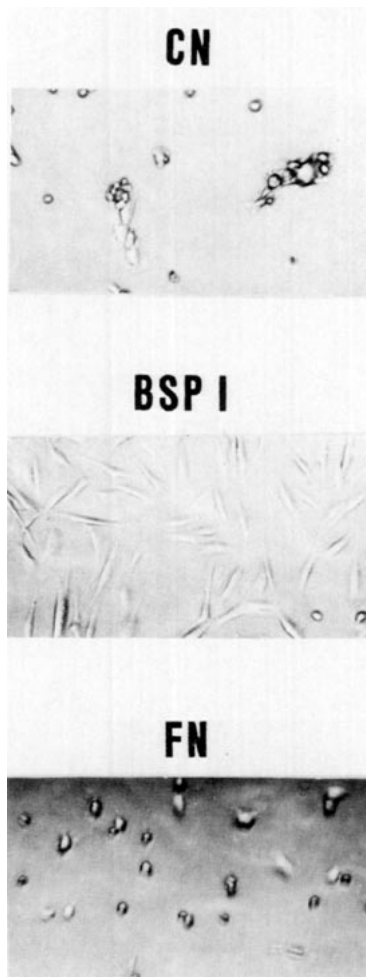


FIGURE 2. Fibroblast attachment and spreading on uncoated dishes (CN) or dishes coated with BSP I or fibronectin (FN) at 24 hr.

with other attachment proteins. BSP I has a persistent effect on cell attachment. BSP I promotes fibroblast cell attachment and spreading for more than 8 days (18). In contrast, cells exposed to BSP II or fibronectin, while demonstrating enhanced attachment and spreading short term (up to 6 hr) exhibit a rounded appearance, associated with decreased cell attachment by 24 hr (Fig. 2). In addition, while fibronectin enhances fibroblast attachment on collagen substrata, BSP I has no effect beyond that observed with cells exposed to collagen-coated dishes (16). To date a collagen domain has not been identified for BSP I, suggesting that in addition to BSP I, other molecules are involved in attachment of osteoblastic cells to osteoid, a predominantly type I collagen matrix. Furthermore, fibronectin promotes the migration of gingival fibroblast, while BSP I does not stimulate cell migration beyond

that observed with control cells (80). At present it is not known whether BSP II or BAG-75 have migration activity.

Thus, the persistent effect of BSP I on cell attachment may in part be related to its inability to promote cell migration. In attempts to better understand the mechanisms regulating BSP I-mediated cell attachment, we evaluated protein production by fibroblasts after exposure to BSP I under normal tissue culture conditions or when exposed to 45°C temperature. A potentially important finding is that fibroblasts grown on BSP I-coated dishes exposed to 45°C temperature for 30 min maintain attached and spread appearance. In contrast, a significant number of cells grown on fibronectin-coated dishes become rounded and/or detached after the 45°C treatment (Fig. 3). In addition, over a 24 hr time period, cells grown on BSP I-coated dishes exhibit enhanced total protein production when compared with cells grown on fibronectin-coated or tissue culture dishes (unpublished data). In support of these findings, previous studies by Polla et al. (81) showed the 1,25-dihydroxyvitamin D<sub>3</sub>, a hormone now known to increase the production of BSP I in bone cells, maintains the adherence of monocytes to tissue culture dishes and protects them from thermal injury. Furthermore, Gronowicz et al. (82) reported that 1,25-dihydroxyvitamin D<sub>3</sub> had pronounced effects on cell shape in osteoblastic cells, probably via *de novo* protein synthesis. The evidence that 1,25-dihydroxyvitamin D<sub>3</sub> can modulate the functions of mononuclear cells (83,84) together with the ability of this hormone to increase the production of BSP I in certain cells suggests that 1,25-dihydroxyvitamin D<sub>3</sub> may, in part, be exerting its effects on cell adherence by stimulating the production of BSP I.

Recently, Gorski and Shikizu (14) extracted and purified a protein from rat bone, bone acidic glycoprotein-75

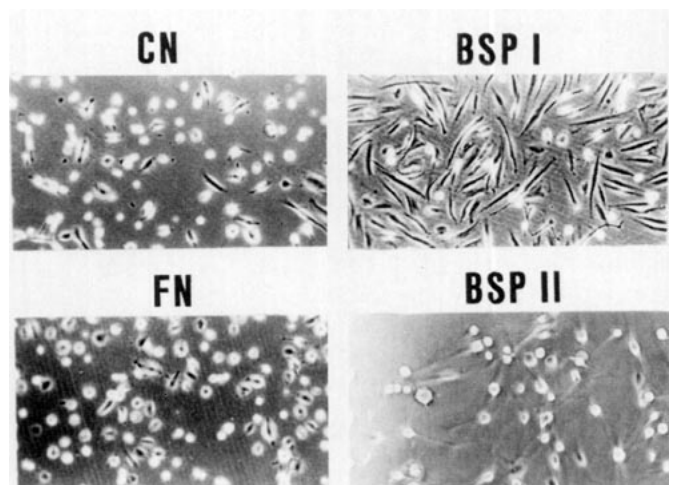


FIGURE 3. Effect of substrata on response of fibroblasts to heat stress. Cells were allowed to attach on tissue culture dishes (CN), or dishes coated with BSP I, BSP II, or fibronectin (FN) for 24 hr, and then cells were exposed to 45°C temperature for 30 min.

(BAG-75) which is a 75,000 molecular weight phosphoprotein containing 7% sialic acid. *N*-terminal sequence analysis showed that BAG-75 is unique, although a 33% homology to BSP I was noted. In addition, they have shown that this protein promotes osteoblastic adherence (J. P. Gorski, personal communication).

The question still remains as to the role of these bone-associated attachment proteins in the regulation of bone homeostasis, as well as the possible role for these proteins in other tissues. All three of these proteins have a strong affinity for calcium, suggesting a common role for these proteins in the transport and storage of calcium. As to a potential function in attachment of cells at an appropriate site *in vivo*, the question arises as to the need for these attachment proteins in bone. In this regard our *in vitro* studies suggest that BSP I and BSP II function differently, e.g., only BSP I-mediated fibroblast attachment in a persistent manner. Recent investigation points to a correlation between shape and attachment of cells and the nature of the expressed gene products. For example, attachment of cells to a substratum is necessary for protein synthesis (translational control) (85). Thus it is interesting that some attachment proteins, e.g., thrombospondin, promote attachment but not spreading of certain cell types (86). Our findings that BSP I, but not fibronectin or BSP II, persistently promotes cell attachment and spreading suggests a unique and separate function for these proteins. These findings may provide clues as to the function of bone-associated BSP I *in vivo*. For example, in fracture healing after migration of appropriate cells into the wound site, BSP I may allow for significant cell numbers to stay at the site. This would provide increased production of extracellular matrix required for bone formation. Further, the inability of these cells to migrate may allow such cells to be imbedded in the bone matrix to function as osteocytes. Other possible functions for BSP I include maintaining a population of monocytes at an inflammatory site, as well as a role in regulating blood clotting.

Studies directed at understanding the interaction of bone-associated attachment proteins with specific cells and identifying specific cell receptors for these proteins will help to clarify the role of these attachment proteins in the modulation of tissue function.

The importance of calcium in regulating protein-mediated cell attachment suggests that any metals that can substitute (compete) for calcium, e.g., lead, can alter cell-cell and cell-substratum interactions. Another concern is that metals may alter the hydroxyapatite crystal thus altering bone cell attachment to the mineralized matrix. In addition, certain metals may compete with calcium at calcium binding sites and subsequently alter protein function, as well as calcium homeostasis. The effects of lead may not only be seen in mineralized tissues, but also in other tissues where calcium-binding bone-associated proteins, e.g., osteonectin and BSP I, have been identified. Studies addressing such issues are currently being designed in our laboratories.

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